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Male preponderance in early diagnosed type 2 diabetes is associated with the ARE insertion/deletion polymorphism in the *PPP1R3A* locus

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Abstract

Background: The ARE insertion/deletion polymorphism of *PPP1R3A* has been associated with variation in glycaemic parameters and prevalence of diabetes. We have investigated its role in age of diagnosis, body weight and glycaemic control in 1,950 individuals with type 2 diabetes in Tayside, Scotland, and compared the ARE2 allele frequencies with 1,014 local schoolchildren.

Results: Men homozygous for the rarer allele (ARE2) were younger at diagnosis than ARE1 homozygotes ($p = 0.008$). Conversely, women ARE2 homozygotes were diagnosed later than ARE1 homozygotes ($p = 0.036$). Thus, men possessing the rarer (ARE2) allele were diagnosed with type 2 diabetes earlier than women ($p < 0.000001$). In contrast, there was no difference in age of diagnosis by gender in those individuals carrying only the common ARE1 variant. Furthermore, although there was no difference in the frequency between the children and the type 2 diabetic population overall, marked differences in allele frequencies were noted by gender and age-of diagnosis. The ARE2 allele frequency in early diagnosed males (diagnosed earlier than the first quartile of the overall ages at diagnosis) was higher than that found in both later diagnosed males and healthy children ($p = 0.021$ and $p = 0.03$ respectively). By contrast, the frequency in early diagnosed females was significantly lower than later diagnosed females and that found in children ($p = 0.021$ and $p = 0.037$).

Comparison of the male to female ratios at different ages-diagnosed confirms a known phenomenon that men are much more prone to early type 2 diabetes than women. When this feature was examined by the common ARE 1/1 genotype we found that the male to female ratio remained at unity with all ages of diagnosis, however, carriers of the ARE2 variant displayed a marked preponderance of early male diagnosis ($p = 0.003$).

Conclusion: The ARE2 allele of *PPP1R3A* is associated with a male preponderance to early diagnosed type 2 diabetes. Susceptibility to type 2 diabetes in later life is not modulated by the ARE2 allele in either sex.

Background

Subgroups of patients with type 2 diabetes mellitus or features of the metabolic syndrome have been shown to have impaired insulin-dependent whole body glucose uptake [1], the principal component of which is non-oxidative glycogen synthesis in skeletal muscle [2]. Glycogen-synthase (GS) catalyses one of the rate-determining steps in glycogen synthesis in muscle and defective insulin-mediated activation of muscle GS has been found in glucose tolerant, but insulin resistant first-degree relatives of Caucasian type 2 diabetic patients [3]. GS is activated in response to insulin through its net dephosphorylation mediated by inhibition of kinases such as glycogen synthase kinase-3 and activation of glycogen-bound protein phosphatase-1 enzyme in response to insulin [4]. In insulin-resistant Pima Indians decreased activities of both GS and glycogen-associated protein phosphatase-1 in muscle tissue has been demonstrated [5,6] providing a mechanism by which GS activation by insulin is reduced in these subjects.

The most abundant glycogen-associated protein phosphatase-1 (PP-1) derived from skeletal muscle is a heterodimer composed of a 37 kDa ubiquitous catalytic subunit and a 160 kDa glycogen-targeting and regulatory subunit, G_M [7]. PP1 G_M has been shown to play an important role in the regulation of glycogen synthase activity [8] and may participate in the insulin stimulation of glycogen synthesis [9]. The gene encoding G_M (*PPP1R3A*) has been located on chromosome 7 [10] in a region in which genome wide scanning in Pima Indians has found markers linked to quantitative traits related to glucose metabolism. Recently a rare premature stop mutation has been detected which produced severe familial insulin resistance when occurring with an unlinked frame shift mutation in a further candidate gene, *PPARG*. [11]. This digenic inheritance serves as a paradigm for the interaction of variation at diverse locations in the genome contributing to the development of the insulin resistant phenotype. Several common variants of *PPP1R3A* have also been variably associated with features of insulin resistance and type 2 diabetes in different populations [10,12–17]. In particular a 5 bp length insertion deletion polymorphism in the 3'-untranslated region (UTR) of the gene gives rise to a difference in distance between two AT rich elements (ARE), which have been implicated in mRNA stability [18,19]. The deletion variant (allele ARE2) has been found to decrease mRNA half life, and *PPP1R3A* transcript and protein concentrations *in vivo* [10] and displays increased binding of a protein responsible for the increased turnover [20]. Thus, the ARE2 variant has implications for the extent of activation of muscle GS by insulin and hence overall insulin sensitivity. This has recently been confirmed by the deletion of the gene for *PPP1R3A* in mice

which results in a markedly insulin resistant phenotype. [21].

The ARE2 allele frequency has been reported to be higher in young type 2 diabetics than a control population in Japan [12] and higher in early diagnosed than late diagnosed type 2 diabetic Pima Indians [10]. In another study ARE2 was weakly associated with whole body insulin sensitivity, with the allele being associated with a trend of higher fasting insulin levels and lower insulin mediated glucose uptake [15]. In contrast a study of aboriginal Canadians showed that ARE2 homozygotes had a lower 2 hour post challenge plasma glucose in type 2 diabetics and individuals with impaired glucose tolerance [14]. Thus the role of the *PPP1R3A* ARE polymorphism in the susceptibility to type 2 diabetes is not clear.

As previous studies have demonstrated an association of the ARE2 variant of *PPP1R3A* with early diagnosed type 2 diabetes and other glycaemic parameters, we have examined its role in such parameters in a genetic sub-study of the Diabetes Audit and Research in Tayside Scotland (DARTS) [22]. In addition we have used a population of local children (aged 5–8) to determine the local gene frequency of the ARE variants at birth, and have examined the divergence from this baseline frequency within the type 2 diabetic population.

Results

We have determined the frequency of the *PPP1R3A* ARE polymorphism in a large group ($n = 1950$) of approximately equal numbers of men and women with type 2 diabetes in Tayside, Scotland and considered association of genotype with age of diagnosis with type 2 diabetes, glycated haemoglobin and body mass index. 847 (43.4%) were ARE1 homozygotes, 891 (45.7%) ARE1/2 heterozygotes and 212 (10.9%) ARE2 homozygotes. Overall the ARE2 allele frequency was 0.337 (0.322–0.352). ARE genotype demonstrated a strong association with age of diagnosis that was both gene dosage and gender dependent (Table 1 and Figure 1). Thus whilst there was no difference between males and females in the mean age of diagnosis for individuals homozygous for the more common ARE1 allele (56.5 years vs 56.7 years respectively), males carrying the less common ARE2 allele (heterozygotes and ARE2 homozygotes) had an earlier mean age of diagnosis (55.1 years) compared with females carrying the ARE2 allele (58.6 years) a difference of 3.6 years which was highly significant ($p < 0.000001$). Furthermore female ARE2 carriers were on average 2 years older ($p = 0.017$) at diagnosis than female ARE1 homozygotes. On the other hand male ARE2 carriers were 1.5 years younger ($p = 0.046$) at diagnosis compared to male ARE1 homozygotes. Thus males and females who had the ARE2/2 genotype differed in mean age of diagnosis by on average 6.5

Table 1: Characteristics of the Tayside Diabetic population by *PP1R3A* genotype and sex. Parameters are given as corrected means \pm Standard deviation, the corrected means are based on multiple measures through time and are adjusted for age diagnosed, age at which parameter was measured and drug treatment at time of measurement.

Male	ARE1/1	ARE1/2	ARE2/2	p value
N	432	479	111	
Age Diagnosed (years)	56.5 \pm 0.6	55.5 \pm 0.5	†53.2 \pm 1.1	0.008 (0.011*)
BMI (Kg/m²)	29.9 \pm 0.2	29.7 \pm 0.2	29.9 \pm 0.5	NS
GHB (%)	7.6 \pm 0.05	7.5 \pm 0.05	7.5 \pm 0.1	NS
Female	ARE1/1	ARE1/2	ARE2/2	p value
N	415	412	101	
Age Diagnosed (years)	56.7 \pm 0.6	58.4 \pm 0.6	†59.5 \pm 1.2	0.038 (0.2*)
BMI (Kg/m²)	31.6 \pm 0.3	31.2 \pm 0.3	30.3 \pm 0.6	0.021
GHB (%)	7.8 \pm 0.05	7.7 \pm 0.05	7.5 \pm 0.1	0.047

* = p value after inclusion of BMI-at-diagnosis in the linear regression model. †p = 0.0001 for comparison between male and female ARE2 homozygotes (recessive model) p < 0.000001 for a comparison between male and female allele carriers (co-dominant model).

years (3.1–9.5, $p = 0.0001$). Over all there was also a weak association of the rare allele with body mass index after adjustment for age of diagnosis, age of BMI, and treatment, with homozygous ARE2 individuals having a BMI 0.81 lower than ARE1 homozygous individuals ($p = 0.041$). This association was however confined to females (BMI difference = 1.41, $p = 0.021$) with males showing no evidence of a difference ($p = 0.939$). There was also a weak association with glycated haemoglobin in the overall population and again this association was confined to females ARE2 homozygotes who had a glycated haemoglobin on average 1% lower than ARE1 homozygotes ($p = 0.047$).

As we found an association of reduced BMI with genotype in females, and increased BMI is known to be the major risk for type 2 diabetes, we included BMI in our regression model for age of diagnosis. This completely removed any significance in the association (Table 1). Interestingly, inclusion of BMI in the regression model in men did not negate the association with earlier age of diagnosis.

To further explore the association of this polymorphism with gender and age of diagnosis, we compared allele frequencies by gender in individuals diagnosed early and those diagnosed later. Early and later diagnosis were defined as being diagnosed at an age below and above the first quartile of the overall ages at diagnosis respectively. This corresponded to being diagnosed less than or greater than 48.2 years in this population. These frequencies were in turn compared with the background population frequency in a large cohort (495 males, 519 females) of healthy school children from the same region. The overall ARE2 allele frequency in the children was very close to the overall frequency in the diabetic population (Table 2 and figure 2). There was no difference in allele frequency

between genders in the children (ARE1 allele frequency in males = 0.336, 0.307–0.366 vs. females 0.330, 0.302–0.359). As expected while there was no difference in ARE2 allele frequency between males and females diagnosed later (OR 0.927, 0.793–1.084 $p = 0.333$) the frequency in early diagnosed males and females was significantly different (OR 1.59, 1.20–2.12 $p = 0.0009$). The frequency in males was 0.383 (0.342 – 0.423) which was significantly higher (OR 1.24, 1.015–1.51, $p = 0.030$) than the background frequency in the children and in early diagnosed females it was 0.280 (0.237 – 0.324) which was in turn significantly lower (OR 0.78, 0.611–0.991, $p = 0.037$) than the background frequency in the children. The frequency in the women diagnosed early was also significantly lower than the frequency in those diagnosed later (OR 0.740, 0.576–0.947, $p = 0.014$). These frequencies were also similarly different in male type 2 diabetics (OR 1.269, 1.030–1.561 $p = 0.021$).

Finally, analysis of the ratio of males to females in the total DARTS population and the genetic sub-study (Go-DARTS) showed a marked overall preponderance of early diagnosis for males that is very similar to that observed in previous epidemiological studies (figure 3a). This was supported by a χ^2 test for trend, of 129.9, $p < 0.0001$, for the total DARTS population and a χ^2 test for trend of 17.9, $p < 0.0001$, in the genotyped subpopulation. This trend resided exclusively in the ARE2 allele carriers with no such trend in ARE1 homozygotes ($p = 0.003$ for comparison of trends by genotype) (figure 3b).

Discussion

We have demonstrated that the ARE polymorphism of *PPP1R3A* is associated with age of diagnosis in the Tayside type 2 diabetic population and that this association is

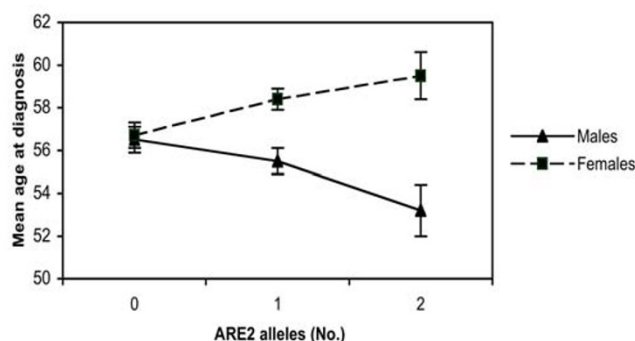


Figure 1
Age diagnosed with type 2 diabetes in males and females by PP1R3A ARE genotype. Legend: Females dashed line open triangle, males solid line closed squares. (Error bars = standard error of the mean)

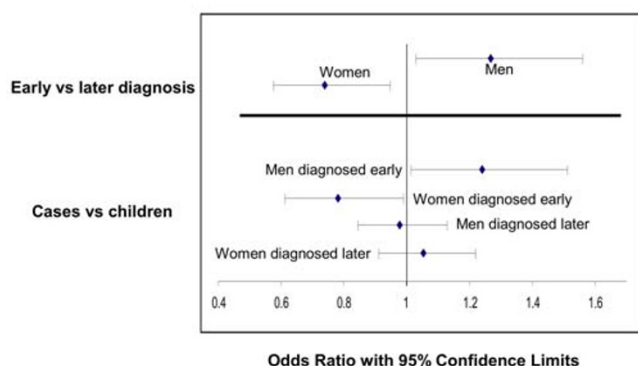


Figure 2
Odds ratios of the ARE2/2 genotype prevalence in type 2 diabetes. Shown are the Odds Ratios and the 95% confidence intervals.

both gender and gene dosage dependent. We know from the United Kingdom Prospective Diabetes Study population that there is a preponderance of males at younger ages of diagnosis and that the proportion of men to women decreased at higher ages of diagnosis [23]. We have reproduced this observation in the DARTS population, however this appears to be confined to ARE2 carriers with no such trend being observed in the ARE1 homozygotes. We have also found a weak association of ARE genotype with BMI and glycated haemoglobin which again appears gender dependent with the association being found exclusively in females. Inclusion of BMI in the model for age of diagnosis attenuated its association with

genotype in the females which suggests that the ARE2 variant is associated with a protection from obesity-induced diabetes in women. The lack of an attenuation in males indicates that the ARE2 allele may predispose men to type 2 diabetes, in a manner independent of obesity.

The overall frequency of the ARE2 variant obtained in the adult population of individuals with type 2 diabetes is almost identical to the frequency found in the children from the same geographical region and these are similar to other published frequencies in Japanese [12], Caucasian [15] and aboriginal Canadian [14] populations. In the study from the Pima Indians however the frequency was found to be considerably higher [10]. In the Japanese population the mean age of diagnosis was similar to the youngest quartile in our population. Examination of the allele frequencies in the men and women diabetics in this population compared with the control population reveals that the risk of diabetes attributable to the variant was predominantly in the males (0.347 vs. 0.288, $p = 0.03$) and was less evident in the females (0.326 vs. 0.288, $p = 0.19$) [12]. This young-diagnosed population also had a male preponderance similar to that observed in our study. The Pima Indian study which examined the relative risk of diagnosis with type 2 diabetes diagnosis before and after the age of 45 also found a significant predominance of the ARE2 variant in the younger age group [10]. In contrast to our study, and that of the Japanese group, this population had a strong female preponderance. Indeed, it is clear that female Pima Indians are not afforded the protection from early type 2 diabetes that is observed in Caucasian populations, and this is likely to be due to the extremely high prevalence of morbid obesity in these women.

There has only been one published study of any significant size that fails to support the involvement of the ARE polymorphism in type 2 diabetes susceptibility. This study is a prospective study of 696 Swedish men that have been followed for 20 years [15]. Although significant effects of the ARE polymorphism on insulin sensitivity were observed, there was no effect of the ARE polymorphism on incident diagnoses of type 2 diabetes. However, this study recruited at age 50 years, and excluded any individuals with a diagnosis of type 2 diabetes or showed evidence of abnormal glucose homeostasis. This means that the prospective Swedish study excluded exactly the population which demonstrated ARE-related risk, both in our study and in the previous Japanese and Pima studies. Indeed, as our study is an unselected population cohort we reconcile the negative results obtained in the Swedish study, in that the ARE polymorphism is not significantly associated with type 2 diabetes risk in the over 50 s. These results are summarised in table 3 and illustrate how different mixes of sex and age at diagnosis in different case-con-

Table 2: Genotype and ARE2 allele frequency (with 95% confidence intervals) in men and women diagnosed with early and late diagnosis of type 2 diabetes.

	Males		Females		Children
	Early	Late	Early	Late	
ARE11	106	326	104	311	457
ARE12	130	349	87	325	438
ARE22	41	70	14	87	119
Total	277	745	205	723	1014
ARE2 frequency	0.383(0.342–0.423)	0.328(0.304–0.352)	0.280(0.237–0.324)	0.345(0.321–0.370)	0.333(0.313–0.354)

Table 3: Concordant relationships between this and previous studies regarding ARE variants and type 2 diabetes

Age at diagnosis	Study	Sample size (n)	Ethnicity	Sex imbalance	Association of ARE variants with diabetes
Younger	Maegawa et al [12]	426	Japanese	Yes	Yes
	Xia et al [10]	930	Pima	Yes	Yes
	This study	482	Caucasian	Yes	Yes
Later	Hansen et al [15]	696	Caucasian	Yes	No
	This study	1,468	Caucasian	No	No

All studies with >200 diabetic individuals are included, 2 studies were excluded due to small sample size and lack of information regarding age of diagnosis [14,29]. Both of these studies did present significant associations with either disease susceptibility or insulin homeostasis.

trol studies may modulate observed associations with ARE2 and susceptibility to type 2 diabetes.

It is not possible from the present study to determine the physiological basis of the differences in males and females observed by *PPP1R3A* genotype although it is probable that the differing hormonal environments in pre-menopausal females is a contributing factor. The mean age of menopause in Caucasian women is 50.5 years and the frequency of the ARE2 allele in women was found to rise steadily to about this age after which it plateaus [data not shown] to a similar level to that found in the children. It is known that women generally have improved glucose tolerance and increased skeletal muscle sensitivity compared to men [24–26]. Recently it has been demonstrated in both rats and humans that lipid infusions lead to insulin resistance in males but not in females. [27,28] It could be speculated that this variant of the *PPP1R3A* gene plays a role in determining response to the high dietary fat intake in the east of Scotland differentially between males and females although the mechanism for this is obscure at present.

Conclusions

The ARE polymorphism has been associated with susceptibility to type 2 diabetes in several case-control studies. We have shown for the first time using a population-based cohort of unselected individuals with type 2 diabetes that

the ARE2 allele is associated with gender selective modulation of the age of diagnosis of type 2 diabetes. This work supports the initial findings that the ARE2 variant modulates susceptibility to type 2 diabetes and indicates an avenue of further investigation for determining physiological basis of gender specific differences in peripheral insulin resistance and onset of type 2 diabetes.

Methods

Tayside Type 2 Diabetic Population

In Tayside, Scotland detailed clinical information on all individuals with diabetes mellitus is recorded on an electronic clinical information system known as DARTS (Diabetes Audit and Research in Tayside Scotland) which has been described elsewhere [22]. In brief, electronic record linkage techniques have identified all people with diabetes in the population of Tayside with a sensitivity of 97%. All clinical data are recorded according to a standard dataset and all case records are validated by a team of dedicated research nurses who create a "cradle to the grave" electronic record. Thus age of diagnosis is rigorously retrospectively determined to coincide with age of onset. All such patients are randomly invited to participate in a genotyping initiative. Following written informed consent, a single sample of blood is collected for DNA extraction and genotyping and the individual assigned a unique system code number for anonymisation purposes. Full compliance with NHS data protection and encryption

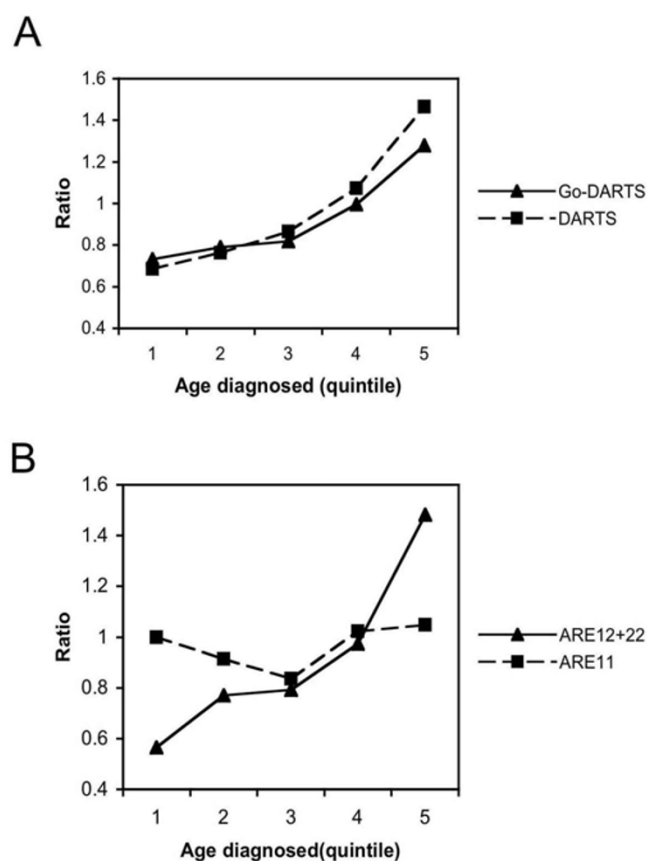


Figure 3
Male preponderance in early diagnosis is associated with the ARE2 variant. A. Ratio of females to males in the total DARTS type 2 population (n= 8155) and in the genotyped subgroup (Go-DARTS, n = 1950) by quintile of age diagnosed B. Ratio of females to males in Go-DARTS by ARE genotype by quintile of age diagnosed. Quintiles were used to maintain equal numbers of individuals in each category and are defined as follows: DARTS: 1=under 48 yrs; 2, 48–56 yrs; 3 = 57–63 yrs; 4 = 64–70 yrs; 5= over 70 yrs. Go-DARTS: 1= under 45 yrs; 2 = 45–54 yrs; 3 = 55–60 yrs; 4 = 60–67 yrs; 5, = over 67 yrs.)

standards is maintained. Genotype information is stored together with the anonymised clinical information on an SQL database. The population in this study therefore consisted of a free-living clinical population selected only on the basis of having type 2 diabetes and attending a diabetes clinic in Tayside. All subjects were of Caucasian ethnicity. All recorded measures of BMI and percentage glycated haemoglobin together with age at diagnosis for each individual was extracted from the DARTS database.

Tayside Children Population

Healthy school children recruited for a gene/nutrition interaction study in the Tayside region of Scotland were used as a control population in this study. They were >95% Caucasian and selected at random from local primary schools. Following informed parental consent mouthwash samples were collected for DNA analysis.

DNA analysis

PPP1R3A ARE polymorphism was analysed using TaqMan-based allelic discrimination assays on a Applied Biosystems 7700 Sequence Detection System. The oligonucleotides used for this assay were:

Primer 1; CAGATAAAACATGGACAATGGCAG.

Primer 2; GGTTGAAATATTTGATCAATGAATCCTG.

ARE1 PROBE (FAM/TAMRA Labelled):

TGGTCTTTTAGTATTGAACATGAAATTTGTATTTAACACTGTATC.

ARE2 PROBE (TET/TAMRA Labelled)

;TGGTCTTTTAGTATTGAACATGAAATTTGTTCAATTTATCATTTA

Assays were performed using reagents and conditions as supplied by the manufacturer (Applied Biosystems, Foster City, CA)

Statistics

Allele frequencies were compared using Pearson's Chi Square. Linear regression was used to assess the affect of genotype on Age of Diagnosis. Repeated measures of Body Mass Index and Glycated haemoglobin were used in a repeated measures model which included age diagnosed, age at which parameter was measured and a term for whether the parameter was measured while the individual was on or off drug therapy for type 2 diabetes including insulin. These parameters were normalised by Log10 transformation. All statistical analyses were performed using algorithms available in turbocharged STATA v7. For consideration of gene frequencies by age diagnosed the population was analysed by quartiles and early diagnosis was classified as being within the first quartile of age diagnosed. Chi squared test for trend was used to examine the gender ratios by quintile and logistic regression analysis was used to compare relative frequencies of the females to males over the quintiles by genotype. All ranges stated in the text are 95% confidence intervals.

Authors' contributions

AD and CP performed the data analysis, BF performed the genotyping, JC recruited the children and collected the mouthwash samples, DB programmed the electronic link-

age tools used for the genetic analysis of the Tayside diabetic population, AM, PC, GL and CP devised and initiated the study. The paper was written by AD and CP with help from all the authors.

All authors read and approved the final manuscript.

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